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SIMULTANEOUS PRODUCTION OF TWO CAPSULAR POLYSACCHARIDES BY PNEUMOCOCCUS

II. THE GENETIC AND BIOCHEMICAL BASES OF BINARY CAPSULATION*

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In the preceding paper, the isolation and some characteristics of a pneumococcus manifesting binary capsulation were described (1). This organism, which was shown to produce two capsular polysaccharides, was derived by transformation of a non-capsulated mutant of pneumococcus Type III with deoxyribonucleates (DNA) of pneumococcus Type I. A noteworthy property of the SI-III cell is the production by it of approximately normal amounts of Type III polysaccharide in contrast to the markedly diminished or absent production of this substance by the cell from which it was derived. Two hypotheses to account for the restitution of the synthesis of Type III polysaccharide by the cell manifesting binary capsulation are readily apparent. Either that part of the genome controlling synthesis of Type III polysaccharide has been restored to normal in the process of cellular transformation to the binary capsular state or the increased production of Type III polysaccharide results from the simultaneous presence within the cell of the mutated Type III capsular genome and the normal Type I capsular genome. Distinction between these two hypotheses may be made by a study of genetic properties of DNA from the SI-III cell. In experiments in which DNA from cells with a binary capsule was applied to non-capsulated organisms derived from a strain of pneumococcus Type II, cells of the mutant $S\text{-}_{III}$ phenotype, SI cells and, rarely, SI-III cells were recovered. No organisms of the SIII phenotype have ever been observed in an experiment of this kind. It may be concluded, therefore, that the augmented production of Type III polysaccharide by the SI-III cell results from the simultaneous pres-

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ence within the cell of the mutated Type III capsular genome and the normal Type I capsular genome rather than from reversion to normality of the mutated Type III capsular locus. From this finding, it follows that there is a discrepancy between the genotype and the phenotype of the SI-III cell in so far as its Type III capsular component is concerned.

To elucidate the mechanism whereby the presence of the Type I capsular genome brings about augmented synthesis of Type III polysaccharide when resident in the pneumococcal cell together with the mutated Type III capsular genome, a biochemical reaction common to the synthesis of these two polysaccharides was sought. All pneumococci are believed to produce a somatic polysaccharide, the C polysaccharide, which is apparently a constituent of the bacterial cell wall. This substance is composed of mannose, galactose, and acetylglucosamine (2). The constituents of Type I capsular polysaccharide are known only in part and consist of galacturonic acid, an acetylhexosamine and an uncharacterized moiety (3, 4). Type III capsular polysaccharide is made up of glucuronic acid and glucose (5). The possibility that some relationship might exist between the biosyntheses of the uronic acid components of the two capsular polysaccharides was suggested by these facts and, consequently, an investigation of the uronic acid metabolism of a variety of pneumococcal strains was undertaken.

The importance of uridine nucleotides to the metabolism of uronic acids in mammalian tissues has been shown by a number of investigators (6). Study of several strains of pneumococcus has demonstrated also the probable importance of such nucleotides to the carbohydrate metabolism of these organisms (7, 8). From an analysis of the content and type of uridine nucleotides of a number of pneumococcal variants and from an examination of the enzymes concerned with their formation, it has been possible to establish the probable basis for the augmented synthesis of Type III polysaccharide in cells of the SI-III phenotype and to make several observations pertaining to the structure and behavior of the capsular genome of pneumococcus.

Materials and Methods

Nomenclature of Pneumococcal Variants.—Because of the lack of correspondence of capsular genotype and phenotype both in non-capsulated variants of pneumococcus and in variants with a binary capsule, it is necessary to introduce separate designations for each. The terms descriptive of phenotype with one addendum will be those set forth in the preceding paper. When two capsular genomes coexist within the cell and only one capsular polysaccharide is expressed, the presence of the cryptic genome will be included in parentheses. For example, phenotype SIII(I) contains mutated genomes of both Type I and Type III but expresses only Type III polysaccharide. To describe the capsular genotype, the following conventions will be used. Each enzyme to be considered will be designated by a capital letter when present in its normal state. Mutations characterized by reduced or by absent enzymatic activity will be described by the corresponding letter in lower case. When experimental conditions do not permit determination of the presence or absence of a given enzymatic function, the function

will be enclosed in parentheses. Subscripts in Roman numerals will be employed to indicate the capsular type designation of the strain from which the DNA determining the enzyme was obtained. Superscripts in Arabic numerals will be used to indicate variants with different mutations in the same enzymatic step or cistron (9). The entire capsular genome of a given capsular type will be delineated by vertical bars. When two capsular genomes coexist within the same cell, their characterizations will be separated by a dot.

The enzymes to which reference will be made are designated as follows:

Uridine pyrophosphogalactose-4-epimerase:	*A*
Uridine pyrophosphoglucose dehydrogenase:	*B*
Uridine pyrophosphogalacturonic acid-4-epimerase:	*C*
The undesignated residue of the capsular genome including the polymerizing system:	*P*

An example of the phenotypic and genotypic description of a pneumococcal strain follows. $S-I_1$ has genotype $|B_1C_1^1(P_1)|$. It is a noncapsulated variant of pneumococcus Type I which fails to produce capsular polysaccharide because of its inability to convert the uridine nucleotide of glucuronic acid to that of galacturonic acid. It may be seen that a genetically distinct strain with a mutation affecting the same biochemical function, $|B_1C_1^3(P_1)|$, would give rise to the same phenotype; *i.e.*, $S-I_2$.

For convenience, uridine nucleotides will be designated by the following abbreviations.

Uridine pyrophosphoglucose:	UPPG
Uridine pyrophosphogalactose:	UPPGal
Uridine pyrophosphoglucuronic acid:	UPPGA
Uridine pyrophosphogalacturonic acid:	UPPGalA

Strains of Pneumococcus.—In addition to the strains described in the preceding report, the following organisms were employed.

S−I phenotypes—$S-I_1$: a non-capsulated mutant derived from Strain I41S.
$S-I_2$: a non-capsulated mutant derived from Strain 1D.
$S-I_3$: a non-capsulated mutant derived from Strain SVI.

SIX, SXIV, SXVIII, SXXII, SXXV, and SXXXIII: capsulated strains of Types IX, XIV, XVIII, XXII, XXV, and XXXIII isolated from patients with pneumonia.

Preparation of DNA and Technique of Transformation Reactions.—The methods were those described by MacLeod and Krauss (10).

Extraction and Identification of Uridine Nucleotides of Pneumococcus.—The organisms were grown in 4 liter cultures of Difco brain-heart infusion broth containing 0.1 per cent neopeptone to which 1 per cent glucose had been added after incubation overnight. Lactic acid formed was neutralized with 3 N NaOH and the cells were harvested at the beginning of the stationary phase of the growth curve by centrifugation. Nucleotides were released from formalinized cells by boiling an aqueous suspension followed by freezing and thawing. The filtered lysate was passed through a Dowex-1 Cl' column to which the nucleotides were absorbed. Fractional elution was carried out with dilute HCl and increasing concentrations of NaCl. The eluates were adsorbed with norite, eluted with ethanol, and chromatographed on paper. Glucuronic and galacturonic acid were distinguished by ionophoresis on paper of their borate complexes. Details of the methods employed have been described elsewhere (7, 8).

Isolation of Enzymes Concerned with the Metabolism of Uridine Nucleotides.—Bacteria were grown in 500 cc. cultures of the medium described above and harvested in the logarithmic phase of growth. The cells were disrupted with ballotini in a Mickle disintegrator at 4°C. and the material released was precipitated with 3 M $(NH_4)_2SO_4$. The precipitate was adsorbed and fractionally eluted from celite to obtain the active materials studied. The substrates employed were UPPG, C^{14}-labelled UPPG and UPPGA. End products of the reactions were identified by chromatographic and by ionophoretic methods. The techniques have been reported in full in a previous communication (11).

EXPERIMENTAL

Genetic and Biochemical Properties of Capsulated and Non-Capsulated Variants of Pneumococcus Type III.—Examination of the nucleotides of capsulated strains of pneumococcus Type III shows both UPPG and UPPGA to be present in significant amounts and an active dehydrogenase capable of converting UPPG to UPPGA can be isolated from such organisms. Examination of four non-capsulated mutants of pneumococcus Type III reveals the near or complete absence of UPPGA from their cells, and enzymatic activity resulting in oxidation of UPPG to UPPGA is of a very low order when compared with that of fully capsulated Type III cells. None of these mutants is virulent in mice. An additional attribute of the four non-capsulated variants of pneumococcus Type III is that each can be transformed to the SI-III phenotype with DNA from pneumococcus Type I. This observation indicates that each strain retains its genetic determinants for the polymerization of Type III polysaccharide and is able to carry out this activity when the constituents necessary for it can be provided by the cell. There is no evidence of any kind to suggest that a wild Type I pneumococcus is endowed with the latent ability to synthesize Type III capsular material.

The observations described may be interpreted to indicate that each of the four non-capsulated variants of pneumococcus Type III has a mutation involving the formation of UPPG dehydrogenase by the cell. That loss of enzymatic activity is not the result of a suppressor mutation is evident from the work of Taylor (12) who pointed out that capsulated Type III organisms were never recovered when DNA from non-capsulated mutant Type III cells was applied to non-capsulated cells derived from an heterologous capsular type. Her observations have been confirmed repeatedly in these experiments.

Although the four non-capsulated mutants are phenotypically similar, they are, as shown first by Ephrussi-Taylor (13), genotypically distinct. DNA from each strain will transform cells of any of the three other strains to the normal SIII phenotype and genotype. The relationships among the four strains resemble, therefore, those of non-identical alleles with the genetic lesions occurring at different sites in a single cistron. The data are summarized in Table I.

Genetic and Biochemical Properties of Capsulated and Non-Capsulated Variants of Pneumococcus Type I.—In Table II are set forth the data obtained from the analysis of pneumococcus Type I and of several non-capsulated variants derived from it. It may be seen that capsulated Type I cells produce a nucleotide not present in capsulated Type III cells, namely UPPGalA. As reported previously (14), this nucleotide is derived from the epimerization of UPPGA rather than from the oxidation of uridine pyrophosphogalactose. The biochemical pathways concerned with the formation of uronic acids in pneumococcus are summarized in Fig. 1. Because two enzymatic steps are involved in

the derivation of UPPGalA from UPPG, it was not unexpected that mutants affecting each of these enzymatic capacities would be found. Like the S−$_{III}$ variants described earlier, strain S−$_{I_2}$ has a mutation affecting the formation

TABLE I

Biochemical Properties and Genotypic Description of Normal and Mutated Strains of Pneumococcus Type III

Phenotype	Nucleotide content		Content of UPPG dehydrogenase	Presence or absence of polymerizing system	Genotype
	UPPG	UPPGA			
SIII	+	+	+	+	$\lvert B_{III}P_{III}\rvert$
S−$_{III_1}$	+	−	±	+	$\lvert b^1_{III}P_{III}\rvert$
S−$_{III_2}$	+	−	±	+	$\lvert b^2_{III}P_{III}\rvert$
S−$_{III_3}$	+	−	±	+	$\lvert b^3_{III}P_{III}\rvert$
S−$_{III_4}$	+	−	±	+	$\lvert b^4_{III}P_{III}\rvert$

+, present; −, absent; ±, detectable but reduced markedly.

TABLE II

Biochemical Properties and Genotypic Description of Normal and Mutated Strains of Pneumococcus Type I

Phenotype	Nucleotide content			Enzyme content		Presence or absence of polymerizing system	Genotype
	UPPG	UPPGA	UPP-GalA	UPPG dehydro-genase	Uronic acid epimerase		
SI	+	+	+	+	+	+	$\lvert B_IC_IP_I\rvert$
S−$_{I_1}$	+	+	−	+	−	?	$\lvert B_Ic^1_I(P_I)\rvert$
S−$_{I_2}$	+	−	−	±	+	?	$\lvert b^2_IC_I(P_I)\rvert$
S−$_{I_3}$	+	+	−	+	−	?	$\lvert B_Ic^3_I(P_I)\rvert$

+, present; −, absent; ±, detectable but reduced markedly.

of UPPG dehydrogenase. It is noteworthy, however, that an active uronic acid epimerase is found in the cells of this strain despite the absence of its substrate, UPPGA. Strains S−$_{I_1}$ and S−$_{I_3}$ have mutations affecting the formation of UPPGalA from UPPGA. UPPG dehydrogenase activity is demonstrable in both strains and UPPGA can be isolated from the cells of each. Like

non-capsulated variants of pneumococcus Type III, those of capsular Type I also lack virulence in mice.

DNA from strain S$-_{I_3}$ will transform cells of strain S$-_{I_1}$ to capsular Type I and the two strains appear to represent, as do the S$-_{III}$ variants, non-identical mutations involving a single cistron. Transformation of S$-_{I_1}$ to capsular Type I can be effected also with DNA from strain S$-_{I_2}$ and the relationships are reciprocal; *i.e.*, cells of S$-_{I_2}$ can be transformed to Type I with DNA of the aforementioned strain. These reactions are characterized, however, by recombination of two enzymatic functions and are analogous to a genetic recombination involving two cistrons.

No system of recombination is currently available to test for the intactness of the polymerizing mechanism in the non-capsulated variants of pneumo-

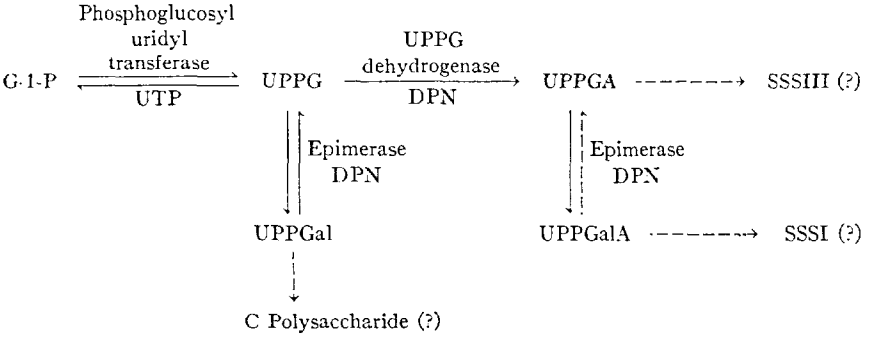

FIG. 1. Pathways of uronic acid metabolism in pneumococcus.

coccus Type I and its status in the strains studied is uncertain. The ability of strain S$-_{I_2}$ to mutate back to the fully capsulated state in certain selective environments suggests that the polymerizing system in this cell is intact, for such reversion to the capsulated state would doubtless be observed infrequently were two mutations required to bring it about.

Some Properties of the Capsular Genome Manifested by Transformation of Non-Capsulated Pneumococci to the Capsulated State.—The data presented so far indicate that the capsular genome of pneumococcus is complex and controls, through the agency of independently variable factors, the execution of a number of biochemical reactions. It is apparent also that when such reactions involve a sequential pathway, a mutation anywhere along that pathway may result in loss of capsulation without affecting unrelated or antecedent biosynthetic reactions. Loss of capsulation, therefore, is not accompanied necessarily by loss or alteration of the entire capsular genome. This assertion is borne out by the fact that a number of non-capsulated pneumococci derived from a variety of capsular types have been found which still produce UPPGA, a non-essential

constitutent for viability of the pneumococcal cell. It is noteworthy that such non-capsulated strains have been derived only from capsulated organisms which include an uronic acid as a constituent of their capsular polysaccharide. Retention of the ability to produce UPPG dehydrogenase, uronic acid epimerase and UPPGA can be used, therefore, as markers of the capsular genome in non-capsulated pneumococci, and the behavior of these markers in transformation reactions can be studied. Previous investigations have demonstrated the absence of uronic acid from the capsular polysaccharides of pneumococcus Types XIV and XVIII (15, 16). Examination of the cells of these capsular types revealed, in addition, the absence of UPPGA and of UPPG dehydrogenase, findings consistent with the earlier observations. Accordingly, DNA was prepared from strains of pneumococcus Types XIV and XVIII and non-capsulated pneumococci producing UPPGA were transformed to these two capsular types. Analysis of the transformed cells (Table III) shows loss of the

TABLE III

Biochemical Properties of Non-Capsulated Variants of Pneumococcus Types I and II before and after Transformation to Capsular Types XIV and XVIII

Non-capsulated Phenotype	UPPGA	UPPG dehydrogenase	Transformed phenotype	UPPGA	UPPG dehydrogenase
$S-I_1$	+	+	XIV	−	−
			XVIII	−	−
$S-II$	+	+	XIV	−	−
			XVIII	−	−

ability to synthesize UPPGA manifested by the non-capsulated cell before transformation. In an analogous fashion loss of the ability to epimerize uronic acid by the non-uronic acid producing strain $S-I_2$ following transformation to Type III can be demonstrated. The results of these experiments suggest that, in a given pneumococcal cell, a specific region of its genetic structure is occupied by the capsular genome and that the capsular genome of one capsular type present in preparations of DNA replaces in the transformation reaction, in a manner analogous to "crossing over," the normal or mutated capsular genome present in the cell. This type of genetic exchange has been demonstrated previously in transformations affecting mode of cellular separation after division (17) and in the presumed direct transformation of capsulated pneumococci to an heterologous capsular type (18).

Transformation of Pneumococci of the $S-III$ Phenotype with DNA from Type I Pneumococcus.—Transformation of $S-III$ pneumococci with DNA from pneumococcus Type I results in the appearance of two transformed phenotypes: SI and SI-III.

Each of the $S-_{III}$ variants listed in Table I can be transformed to the SI phenotype. DNA from such transformed Type I cells, like that from wild Type I cells, will transform the same $S-_{III}$ variants to the SI and SI-III phenotypes. It is noteworthy, however, that, after any of the $S-_{III}$ variants has been transformed to the Type I phenotype, DNA from it will no longer react with genetically heterologous $S-_{III}$ variants to give rise to Type III pneumococci. The finding indicates that the $S-_{III}$ cell transformed to capsular Type I has lost its mutated Type III capsular genome and has acquired in its stead, the normal Type I capsular genome. In so doing, it has acquired also the ability to carry out three biochemical functions: the conversion of UPPG to UPPGA, the conversion of UPPGA to UPPGalA and the polymerization of Type I capsular polysaccharide.

Transformation to the binary capsular SI-III phenotype occurs also following exposure of $S-_{III}$ cells to DNA from Type I pneumococci. The phenotype and genotype of an SI-III strain are shown in Table IV. Evidence supporting this structure for the genotype of the SI-III strain is derived from study of the phenotypes and genotypes obtained following transformation of a non-capsulated mutant of pneumococcus Type II with DNA from SI-III cells. Pneumococci of three different phenotypes are recovered: $S-_{III}$, SI, and SI-III, the last being an infrequent finding. It is noteworthy that no SIII cells are observed and all the experimental data indicate that recombination between subunits of the SI and SIII capsular genomes does not take place; *i.e.*, wild type B_I does not correct the alteration b_{III} in the mutated Type III strain by recombining to give the hypothetical genome $| B_I P_{III} |$. Each capsular genome in the wild type or mutated state behaves as a tightly linked unit composed of recognizable subunits controlling a variety of biochemical functions.

It is apparent also from examination of SI-III cells that this variant produces an approximately normal amount of Type III capsular polysaccharide despite the absence from it of the normal Type III capsular genome. In addition, the amount of Type I capsular material, as judged from the quellung reaction, is distinctly less than that produced by most unitypic Type I strains. From an analysis of the capsular genome of the SI-III cell, it may be concluded that the Type I capsular genome functions as an augmenter of Type III capsular polysaccharide synthesis by providing UPPGA and conversely, the mutated Type III capsular genome suppresses partially the synthesis of Type I polysaccharide by competing for an intermediary metabolite essential for its formation.

Further support for these interpretations may be had from an examination of the behavior of $S-_{III}$ cells exposed in the transforming system to DNA from different non-capsulated strains derived from capsular Type I. From the data in Table IV it is apparent that DNA of non-capsulated variants of pneumococcus Type I which lack uronic acid epimerase but continue to oxidize

UPPG to UPPGA are capable of transforming S$-_{III}$ cells with a resultant increase in the formation of Type III polysaccharide. Although these cells produce no Type I polysaccharide because of their inability to form UPPGalA from UPPGA and are phenotypically indistinguishable from strains of SIII, genotypically they are related closely to strains of the SI-III phenotype and are designated SIII(I) because the mutated Type I capsular genome is carried

TABLE IV

Phenotypic and Genotypic Description of Pneumococci before and after Transformation

Receptor cell		Source of DNA		Transformed cell	
Phenotype	Genotype	Phenotype	Genotype	Phenotype	Genotype
S$-_{III_4}$	$\|b^4_{III}P_{III}\|$	S$-_{III_2}$	$\|b^2_{III}P_{III}\|$	SIII	$\|B_{III}P_{III}\|$
		SI	$\|B_IC_IP_I\|$	SI	$\|B_IC_IP_I\|$
				SI-III	$\|b^4_{III}P_{III}\|\cdot\|B_IC_IP_I\|$
		S$-_{I_1}$	$\|B_Ic^1_I(P_I)\|$	SIII(I)	$\|b^4_{III}P_{III}\|\cdot\|B_Ic^1_I(P_I)\|$
		S$-_{I_2}$	$\|b^2_IC_I(P_I)\|$	S−	Not examined
S$-_{II}$	$\|B_{II}(P_{II})\|$	SI-III	$\|b^4_{III}P_{III}\|\cdot\|B_IC_IP_I\|$	S$-_{III_4}$	$\|b^4_{III}P_{III}\|$
				SI	$\|B_IC_IP_I\|$
				SI-III	$\|b^4_{III}P_{III}\|\cdot\|B_IC_IP_I\|$
S$-_{III_2}$	$\|b^2_{III}P_{III}\|$	SIII(I)	$\|b^4_{III}P_{III}\|\cdot\|B_Ic^1_I(P_I)\|$	SIII	$\|B_{III}P_{III}\|$
S$-_{I_2}$	$\|b^2_IC_I(P_I)\|$	SIII(I)	$\|b^4_{III}P_{III}\|\cdot\|B_Ic^1_I(P_I)\|$	SI	$\|B_IC_IP_I\|$
S$-_{I_1}$	$\|B_Ic^1_I(P_I)\|$	S$-_{I_2}$	$\|b^2_IC_I(P_I)\|$	SI	$\|B_IC_IP_I\|$
		S$-_{I_3}$	$\|B_Ic^3_I(P_I)\|$	SI	$\|B_IC_IP_I\|$

cryptically. DNA from an SIII(I) strain applied to an heterologous S$-_{III}$ strain will transform it to the Type III phenotype and genotype. The same preparation of DNA will transform an heterologous S-$_I$ strain to the Type I phenotype and genotype. The results are in accord with those described by Hotchkiss (19) concerning the "repair" of alterations in the maltase locus in pneumococcus. "Small" alterations are restored to the normal state through transformation more readily than are "large" ones. In capsular transformations, restoration to wild type by intratype transformation occurs in most instances

at a higher frequency than does transformation to heterologous or to binary capsular types.

Non-capsulated strains of pneumococcus Type I which, in contradistinction to those employed in the previous experiments, have lost their ability to synthesize UPPGA possess DNA which is incapable of leading to augmentation of Type III polysaccharide synthesis on the part of the $S-_{III}$ cell. Because neither the cell to be transformed nor that serving as a source of DNA can produce UPPGA, the result of such experiments is the anticipated one.

In transformation reactions involving cells of $S-_{III}$ phenotypes and the non-capsulated variant of pneumococcus type I, $S-_{I_1}$, which produces UPPGA, cells of each strain may function as the donor of or as the recipient of the DNA of the heterologous capsular genome. Although $S-_{III}$ cells may accept the capsular genome of wild or of mutated variants of pneumococcus Type I, no strains have been isolated in which a normal or mutated Type I capsular genome coexists in the cell with a normal Type III capsular genome. Whether or not the latter relationships can be established cannot be ascertained from available data; but, if they are possible ones, their occurrence must be infrequent.

The Ability of DNA of Pneumococci, the Capsular Polysaccharides of Which Contain Uronic Acid, to Give Rise to Binary Capsulation.—It has been recognized for a number of years that uronic acids are constituents of polysaccharides of pneumococci of several capsular types (20, 21). If the interpretations of the phenomenon of binary capsulation set forth above are correct, it is reasonable to anticipate that DNA from capsulated pneumococci other than that from pneumococcus Type I might give rise to variants manifesting binary capsulation. In Table V are recorded the results of transforming four genotypically distinct variants of the $S-_{III}$ phenotype with DNA from a variety of capsulated pneumococci each of which includes an uronic acid in its capsular polysaccharide. It may be seen that cells of three additional phenotypes with binary capsules have been isolated: SIII-IX, SIII-XXV, and SIII-XXXIII. Although augmentation of the synthesis of Type III polysaccharide is clearly manifested by cells of each of these phenotypes, in none is it as pronounced as by cells of the SI-III phenotype. Colonies of cells of these phenotypes, therefore, may be difficult to distinguish from those of singly capsulated strains. Noteworthy is the fact that, to date, no one of the $S-_{III}$ strains has been found capable of being transformed to all the recognized phenotypes manifesting binary capsulation and also that DNA from some strains of capsulated pneumococci producing uronic acid; *e.g.*, Types II, VIII, XXII, have failed to give rise to the phenomenon of binary capsulation. The explanation of these variations in the behavior of different cell lines is not apparent at the present. The occurrence of phenotypes with binary capsules other than SI-III, however, is in accord with the stated hypothesis concerning the mechanism of augmented synthesis of Type III polysaccharide.

DNA from cells of each of the phenotypes manifesting binary capsulation has been tested in transformation reactions with appropriate receptor strains. In each instance, augmentation of the synthesis of Type III capsular polysaccharide appears to result from the simultaneous presence within the cell of the mutated Type III capsular genome and the normal capsular genome of the second capsular type. In no instance has there been evidence of alteration toward normal of the mutated Type III capsular genome, cells of each phenotype with a binary capsule seeming to be constructed genetically in a fashion analogous to those of the SI-III phenotype.

Utilization of Binary Capsulation as an Indicator of the Presence of Uronic Acid in Capsular Polysaccharide.—If DNA from a capsulated pneumococcus

TABLE V

Phenotypes Recovered Following Transformation of Non-Capsulated Mutants of Pneumococcus Type III with DNA from Capsulated Pneumococci Producing UPPGA

Strain transformed	Source of DNA								
	I	II	III	V	VIII	IX	XXII	XXV	XXXIII
$S-III_1$	I I–III	II	III	—	VIII	IX	XXII	 III–XXV	XXXIII III–XXXIII
$S-III_2$	I I–III	II	III	—	VIII	IX III–IX	XXII	XXV	XXXIII III–XXXIII
$S-III_3$	I I–III	II	III	—	VIII	IX III–IX	XXII	XXV	XXXIII III–XXXIII
$S-III_4$	I I–III	II	III	V III–V	VIII	IX	XXII	XXV III–XXV	XXXIII

gives rise to a phenotype with a binary capsule when allowed to react with cells of an $S-III$ strain in the transforming system, its cells should be capable of synthesizing UPPGA and its capsular polysaccharide should contain an uronic acid. Transformation of strain $S-III_4$ with DNA from pneumococcus Type V has led to the isolation of cells of the SIII-V phenotype. Although the composition of Type V polysaccharide was unknown at the time the SIII-V phenotype was isolated, it was predicted that UPPG dehydrogenase and UPPGA would be present in its cells and an uronic acid would be a constituent of its capsular polysaccharide. Analysis of Type V cells and polysaccharide has confirmed fully these predictions (22).

DISCUSSION

Investigation of certain uridine nucleotides and of the enzymes concerned with their formation in pneumococcus has led to a number of findings of

interest. The relation of the ability to synthesize uronic acids to maintenance of the capsulated state and concomitantly of virulence in certain pneumococcal types is clearly evident. Failure to synthesize UPPGA results in loss of capsulation by pneumococci of Types I and III and the inability to convert UPPGA to UPPGalA causes a similar effect in pneumococcus Type I. It is obvious that mutations affecting metabolic functions other than those related to uronic acids may cause loss of capsulation, for capsulated strains of Types XIV and XVIII, which produce no uronic acids, may mutate to the non-capsulated state. Even types of pneumococci which include glucuronic acid as an important constituent of their capsular polysaccharide may mutate to the non-capsulated state without loss of the ability to form UPPGA. A non-capsulated strain of pneumococcus Type II and two non-capsulated variants of pneumococcus Type VIII have each been found to retain the ability to synthesize the uridine nucleotide of glucuronic acid while failing to produce capsular polysaccharide. It is evident also from these observations that loss of capsulation by pneumococcus does not necessarily entail deletion of the capsular genome but may result from an alteration affecting any one of the several biochemical activities determined by it.

From a study of non-capsulated variants of pneumococcus Type III, the frequency with which mutation alters the activity of UPPG dehydrogenase in such strains is readily apparent. Of eight phenotypically similar, genotypically distinct $S-_{III}$ phenotypes, all have been found to have mutations affecting this biochemical activity. Most of these strains manifest slight but detectable UPPG dehydrogenase activity and behave as "leaky" mutants described in other bacterial species. They retain intact, however, the metabolic capability of polymerizing Type III polysaccharide. This finding does not preclude the occurrence in other non-capsulated variants of pneumococcus Type III of mutations affecting the polymerizing system which may result in loss of capsulation. On the other hand, mutations preventing the formation of UPPG would probably be lethal mutations. Strains of pneumococcus grown in the absence of galactose must form this constituent of their C or cell wall polysaccharide by the epimerization of UPPG to UPPGal. Although mutations in the structure of C polysaccharide have been shown by McCarty (23) to be consistent with cellular viability in Group A beta hemolytic streptococci, mutations of this character have not been described to date in pneumococcus. It is of interest that the mutations affecting the C polysaccharide of Group A streptococcus alter its content of acetylglucosamine, a constituent of pneumococcal C polysaccharide.

Through the investigation of capsulated and non-capsulated variants of pneumococcus Type I, the biochemical pathway for the synthesis of galacturonic acid has been elucidated. Although initially it appeared likely that UPPGalA would be formed by the oxidation of UPPGal because of the pre-

sumed required presence of the latter for the formation of C polysaccharide, such did not prove to be the case. UPPGA was found first in non-capsulated and later in capsulated variants of pneumococcus Type I and an enzyme capable of converting UPPGA to UPPGalA was demonstrable in the latter (14). This pathway for the synthesis of galacturonic acid appears to be widespread in nature, for it has been reported to be present in mung bean seedlings and several other plants (24). It is of probable importance in the synthesis of pectins, which are polygalacturonic acids, as well as in the synthesis of Type I pneumococcal capsular polysaccharide.

Observations on the transformation of non-capsulated pneumococci to an heterologous capsular type raise several questions of interest. From the experimental data, it seems likely that in a given pneumococcal cell, the capsular genome is relegated to a specific region of the total genome. If this is the case, it appears that, when transformation occurs with acquisition of new biosynthetic capabilities and loss of ones possessed previously by the cell, a reaction analogous to "crossing over" has taken place between the two capsular genomes, the one of the cell and that present in the DNA inducing transformation. Such an event presupposes antecedent pairing of the two capsular genomes. In view of the rather marked dissimilarities of biochemical function requisite for the synthesis of different pneumococcal capsular polysaccharides, the question arises whether or not special determinant groupings, not related necessarily to the specific processes of capsular synthesis, may be responsible for such pairing. Such determinants might be DNA or conceivably some other material such as protein. It is of interest that although non-capsulated pneumococci derived from other than Types I and III have been transformed directly to the binary capsular SI-III phenotype with DNA from cells of this binary capsular type, such a transformation is an uncommon event, and segregation of the two capsular genomes is observed more commonly. It is noteworthy, however, that some relationship appears to exist between the two capsular genomes of the cell with a binary capsule, for only DNA from such cells has been observed to transform unrelated non-capsulated variants to the binary capsular state. Mixtures of two DNA preparations each bearing one of the two capsular genomes have failed to yield comparable results. "Linkage" between the mutated Type III genome and the normal Type I genome in cells of the SI-III phenotype, however, is evidently of a low order if, indeed, it truly exists.

Examination of the capsular genome of Type I and of Type III pneumococci shows each to control several biochemical activities concerned with the synthesis of the capsule. In capsular transformation, the genetic factors controlling these several activities appear to reside in a single particle of DNA and to be transferred in intertype transformations as a unit. In the case of the Type I capsular genome, which controls a minimum of three biochemical activities,

the frequency of intertype transformation to capsular Type I effected by DNA from Type I pneumococci is too high to be accounted for on any other basis. In addition, examination of the capsular genome of variants manifesting binary capsulation fails to give any evidence that a subunit of the Type I capsular genome can recombine with the mutated Type III capsular genome resident within the same cell, for normal Type III strains are not recovered in such reactions.

Intratype transformations of two kinds may be discerned from the study of genetic interactions between non-capsulated variants of Type I and between non-capsulated strains of Type III. The capsular genomes of phenotypically similar, genotypically distinct variants of pneumococcus Type I with mutations affecting the activity of uronic acid epimerase may undergo recombination in transformation reactions with restoration of that enzymatic function and of capsular synthesis to normal. In analogous fashion, the capsular genomes of mutant Type III strains with mutations affecting UPPG dehydrogenase may interact. In each instance, these intratype reactions are analogous to those described by Benzer (9) as affecting a single cistron. A second type of intratype reaction may occur between non-capsulated mutants of pneumococcus Type I distinguishable by their different enzymatic alterations. In reactions of this kind, interaction between the capsular genomes of the two variants, one with a mutation affecting UPPG dehydrogenase and the other with one affecting uronic acid epimerase, leads to restoration of the synthesis of Type I polysaccharide. In transformations of this kind, two "cistrons" are involved. Because of the absence of suitable selective techniques, information is lacking concerning segregation of the genetic subunits of a given capsular genome. The data relating to binary capsulation derived from the strains described herein indicate that, if such divisions of the capsular genome do occur, they do not lead to the formation of structures which participate in intertype reactions.

Because of the nature of the transformation reaction, it has not been possible to determine with assurance whether or not recombination between the genome of the cell and that taken up from its environment has occurred as a result of physical exchange of DNA or of copy choice. A third mechanism of genetic variation is suggested by experiments of MacLeod and Krauss (25) wherein non-capsulated mutants of pneumococcus Type VIII are transformed to capsular Type VIII by DNA from organisms of heterologous capsular type. The results are compatible with the view that introduction into the cell of certain heterologous particles of DNA may alter the stability or mutability of a given locus. Such an hypothetical event might be considered analogous to the phenomena of conversion described by Lindegren (26) and by Mitchell (27) and of paramutation reported by Brink (28). In view of the apparent failure of the units controlling the synthesis of UPPG dehydrogenase to recombine in intertype transformation involving pneumococcus Types I and

III, there arises the question of whether or not recombination takes place in intratype transformations involving the cistron controlling this enzyme. At present, the possible influence of a capsular genome taken up by the cell upon that already resident within the genome of that cell cannot be excluded. Such an event would provide a plausible, though unproved explanation of the several phenomena described.

Binary capsulation in pneumococcus provides an example of a competitive biochemical reaction with one metabolic pathway vying for an intermediary product of another. The intracellular relationships are such that in the SI-III phenotype, the mutated Type III capsular genome acts as a suppressor of the end product of the Type I capsular genome and conversely, the latter functions to augment expression of the phenotype controlled by the former. Here genetic suppression and augmentation can be defined in biochemical terms. These findings may be somewhat analogous to those resulting in the formation of Sab phenotypes of *H. influenzae* when R_b cells are transformed with DNA from Sa strains of the same species (29). The biochemical basis for the latter reaction is at present undefined. Another interesting feature of the pneumococcal phenotype manifesting binary capsulation is the fact that it permits recognition of the persistence within the non-capsulated variants of pneumococcus Type III of the biochemical system requisite for the polymerization of Type III polysaccharide. This capacity of the mutant Type III strains cannot be identified at the present time by biochemical techniques but can be demonstrated through genetic interactions. Analogous recombinations permitting recognition of the capsular polymerizing systems of other non-capsulated pneumococci have not yet come to light.

Definition of the biochemical relationships existent within the SI-III cell have permitted predictions concerning genetic interactions which might lead to the formation of other binary capsular phenotypes; and, in three instances, such predictions have been realized with the formation of additional types of cells manifesting binary capsulation: SIII-IX, SIII-XXV, and SIII-XXXIII. Why DNA from other capsulated strains of pneumococci which produce uronic acids fails to give rise to the phenomenon of binary capsulation is not understood at the present time.

In a fashion analogous to the use of serologic cross-reactions to obtain data indicative of the structure of polysaccharides (30), transformation reactions giving rise to binary capsulation may be employed. Although the structure of Type V pneumococcal polysaccharide was unknown, DNA from Type V strains was found to give rise to cells of the SIII-V phenotype in transformation reactions with an $S-_{III}$ variant. On the basis of this finding, it was predicted that UPPG dehydrogenase and UPPGA would be present in the cells of Type V pneumococci and that Type V capsular polysaccharide would have an uronic acid as one of its components. These predictions have been confirmed (22).

Study of binary capsulation in pneumococcus has brought to light a number

of facts related to the genetic and biochemical properties of this organism. Further study of the phenomenon should provide additional data of general biological interest.

SUMMARY

Study of the capsular genome of pneumococcus has shown that it controls a multiplicity of biochemical reactions essential to the synthesis of capsular polysaccharide.

Mutation affecting any one of several biochemical reactions concerned with capsular synthesis may result in loss of capsulation without alteration of other biochemical functions similarly concerned.

Mutations affecting the synthesis of uronic acids are an important cause of loss of capsulation and of virulence by strains of pneumococcus Type I and Type III.

The capsular genome appears to have a specific location in the total genome of the cell, this locus being occupied by the capsular genome of whatever capsular type is expressed by the cell.

Transformation of capsulated or of non-capsulated pneumococci to heterologous capsular type results probably from a genetic exchange followed by the development of a new biosynthetic pathway in the transformed cell. The new capsular genome is transferred to the transformed cell as a single particle of DNA.

Binary capsulation results from the simultaneous presence within the pneumococcal cell of two capsular genomes, one mutated, the other normal. Interaction between the biochemical pathways controlled by the two capsular genomes leads to augmentation of the phenotypic expression of the product controlled by one and to partial suppression of the product determined by the other.

Knowledge of the biochemical basis of binary capsulation can be used to indicate the presence of uronic acid in the capsular polysaccharide of a pneumococcal type the composition of the capsule of which is unknown.

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